Antiviral Research 82 (2009) 148–156

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

New tools to study RNA interference to fish viruses: Fish cell lines permanently expressing siRNAs targeting the viral polymerase of viral hemorrhagic septicemia virus

S. Ruiz [d], B.D. Schyth [c], P. Encinas [d], C. Tafalla [a], A. Estepa [b], N. Lorenzen [c], J.M. Coll [d,*]

[a] Instituto Nacional Investigaciones Agrarias, INIA, CISA – 28130 Madrid, Spain
[b] University Miguel Hernandez UMH, IBMC, 03202, Elche, Spain
[c] The National Veterinary Institute, Hangøvej 2, 8200 Århus N, Denmark
[d] Instituto Nacional Investigaciones Agrarias, INIA, Biotecnología.Crt. La Coruña, Km. 7, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 15 September 2008
Received in revised form 19 February 2009
Accepted 25 February 2009

Keywords:
siRNA
VHSV
Polymerase
Fish cell lines
EPC
Hairpin

ABSTRACT

Previous studies have indicated that low transfection efficiency can be a major problem when gene inhibition by the use of small interfering RNAs (siRNAs) is attempted in fish cells. This may especially be true when targeting genes of viruses which are fast replicating and which can still infect cells that have not been transfected with the antiviral siRNAs. To increase the amount of antiviral siRNAs per cell a different strategy than transfection was taken here. Thus, we describe carp epithelioma papulosum cyprinid (EPC) cell clones expressing siRNAs designed to target the L polymerase gene of the viral hemorrhagic septicemia virus (VHSV), a rhabdovirus affecting fish. Eight siRNA sequences were first designed, synthesized and screened for inhibition of *in vitro* VHSV infectivity. Small hairpin (sh) DNAs corresponding to three selected siRNAs were then cloned into pRNA-CMV3.1/puro plasmids, transfected into EPC cells and transformed clones were obtained by puromycin selection. Sequence-specific interference with VHSV could only be observed with EPC clones transformed with a mixture of the three shDNAs, rather than with those clones obtained with individual sh DNAs. However, interference was not specific for VHSV as infection with an heterologous fish rhabdovirus, was also reduced to a similar extent. It was shown that this reduction was not due to an Mx response in the transformed cell clones. Here, we discuss some of the possible reasons for such data and future work directions. EPC clones stably expressing rhabdoviral specific siRNA sequences could be a strategy to further investigate the use of RNA interference for targeting costly fish pathogenic viruses.

1. Introduction

The lack of a licensed vaccine against fish rhabdoviruses viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV) or Spring viremia of carp virus (SVCV) (Lorenzen and LaPatra, 2005), together with the high mortalities they produce, even in adult fish, place these pathogens as responsible for the most damaging diseases of international aquaculture. Therefore, any further understanding of the determinants of their virulence and/or replication mechanisms might help to design more effective methods of prevention.

Fish rhabdoviruses, such as VHSV, are composed of an exterior lipid membrane envelop projecting trimeric spikes of their glycoprotein G (Coll, 1995) and an interior nucleocapsid wrapping a single stranded RNA of negative polarity of 11,000 nucleotides. Their whole sequenced genome codes for the structural L, G, N, M1 and M2 viral proteins (Heike et al., 1999) and the Nv non-viral protein (Kurath et al., 1995). The polymerase L (190 kDa), associated with the viral RNA in the nucleocapsid, contains their transcriptase and replicase activities (Wagner, 1987). Once inside the cytoplasm, the L polymerase replicates the rhabdoviral genome via a full length positive sense strand forming double stranded RNA intermediates and also serving as a template to synthesize more negative genome strands and the rest of proteins to complete the new rhabdoviral protein particles (Purcell et al., 2006).

During the last years, double stranded RNAs of 19–25-nucleotides (small interfering RNA or siRNA) have been shown to inhibit translation of complementary sequence messenger RNAs (mRNAs) and in some cases they also induce target RNA cleavage. This phenomenon is being applied to many biological model species to specifically knock down gene expression (Caplen, 2003; Colbère-Garapin et al., 2005). Some examples of mammalian viral replication inhibition by using siRNA with complementary sequences to their polymerases include HIV-1 (Novina et al.,

* Corresponding author. Tel.: +34 1 3476850; fax: +34 1 3572293.
E-mail address: juliocoll@inia.es (J.M. Coll).

0166-3542/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2009.02.200

2002), respiratory syncytial virus (Li et al., 2005), hepatitis C virus (Baltayiannis et al., 2005; Korf et al., 2005) and vesicular stomatitis virus, a mammalian rhabdovirus (Barik, 2004).

Although the RNA interference (RNAi) technology offers a new way to study fish rhabdoviruses, only a few studies have been published on such methods in fish (Schyth et al., 2006, 2007). However, in those studies problems were experienced including low transfection efficiencies and both siRNA and transfection agent-related Mx/interferon induction (Schyth et al., 2006, 2007). Previous attempts to explore RNAi in the zebrafish model reported only non-specific effects (Gruber et al., 2005). As fish cells are commonly hard to transfect, siRNA transfection efficiency may be one of the main problems in the cause of gaining a good target gene reduction (Gruber et al., 2005). Further, because siRNA can be endogenously expressed in mammalian cells from eukaryotic plasmids containing small hairpin (sh) siRNA-generating DNA sequences (two complementary DNA sequences of 21 nucleotides separated by a loop of 9 nucleotides producing shRNA structures) (Xia et al., 2003), we explored the possibility of obtaining fish cells with a stable production of rhabdoviral sequence-specific siRNA. Thus, plasmids including the necessary sh DNA precursor sequences under the control of the human cytomegalovirus promoter (Xia et al., 2003) were used to transform epithelioma papulosum cyprinid (EPC) cells from carp. The use of plasmid vectors including the puromycin resistance gene allowed to select for EPC clones permanently transformed with the above mentioned plasmids. Such clones were stably producing specific siRNAs and interfering with VHSV/IHNV replication with clone-dependent variation.

2. Materials and methods

2.1. Viruses

The VHSV 07.71 isolated in France (LeBerre et al., 1977) from rainbow trout *Onchorynchus mykiss* (Walbaum) was grown and assayed for infectivity in EPC cells as described (Basurco et al., 1991). The IHNV used was the isolate CSF 220-90 (LaPatra et al., 1994).

2.2. Design, selection and synthesis of VHSV specific siRNA

The mRNA corresponding to the gene of the L polymerase was obtained from the complete genome sequence of VSHV 07.71 (Gene Bank accession numbers NC000855 and AJ233396). By using 12 different programs (Ambion, Jack, OptiRNAi, Emboss-2.9.0, MPI principles, GenScript, Dharmacon, Qiagen, siDirect, InVitroGen, Deqor and MPI + rational) for siRNA design (available at http://i.cs.hku.hk/~sirna/software/sirna.php), siRNA sequences were designed to target the L polymerase gene of VHSV 07.71 (Table 1). The target sequences were then synthesized as double stranded siRNA by Ambion (Austin, TX). Fluorescently labelled non-specific siRNA used as controls to estimate transfection efficiencies and/or controls for the immunodetection focus assay were o121, a siRNA specific for GFP (CGGCAAGCTGACCCTGAAGTTCAT) and o122, a siRNA labelled with Alexa fluor 488 siRNA (AATTCTCCGAACGTGTCACGT), both from Qiagen (catalog nos. 1022064 and 1022563, respectively).

2.3. Transfection of EPC cells with siRNAs

Each of the siRNA (Table 1) was transfected as previously described (Schyth et al., 2006), except by the use of RPMI 1640 medium Dutch modification (Sigma Chemical Company), gassing with 5% CO_2 and incubating 24 h post-transfection before the assay (Rocha et al., 2004). Briefly, different concentrations of each siRNA in 10 μl per well were incubated with 25 μl of RPMI per well containing 1 μl per well of previously diluted TransIT-TKO from Mirus (Euromedex, Souffelweyersheim, France) during 20 min. The mixtures were then added to EPC cell monolayers with 100 μl of complete cell culture medium per well, the cultures gassed with 5% CO_2 in air and the mixtures incubated during 1 h at 37 °C and then for 24 h at 28 °C. Before VHSV infection, the EPC cell monolayers were washed with RPMI to avoid interference of the TransIT-TKO reagent with the virus.

2.4. Immunodetection of VHSV-infected foci by anti-N MAb in EPC cell monolayers

The EPC cell monolayers were infected at 14 °C during 2 h with gentle agitation with 2×10^3 focus forming units (ffu)/ml of VSHV 07.71. Then the EPC cell monolayers were washed with 100 μl/well of cell culture medium with 2% FCS and incubated overnight at 14 °C. The monolayers were then fixed with cold methanol during 10 min and air dried. To detect the N protein of VHSV, the MAb 2C9 (Sanz and Coll, 1992a) 1000-fold diluted in dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.24 mM merthiolate, 5 g of Tween 20/l, 50 mg of phenol red/l, pH 6.8) was added to the wells (100 μl/well) and incubated for 1 h at room temperature. After washing with distilled water, 100 μl of peroxidase-labelled anti-mouse IgG (Nordic, Tilburg, The Netherlands) were added per well and incubation continued during 30 min (Sanz and Coll, 1992a). After 3 washings by immersion in distilled water, 50 μl of 1 mg/ml of diaminobenzidine (DAB) per well (Sigma) in the appropriate buffer were added, and the reaction allowed to proceed until brown foci were detected by inspection with an inverted microscope in the controls containing no siRNAs (Lorenzo et al., 1996). Once washed with water and air dried, brown foci (DAB-positive foci or ffu) were counted with an inverted microscope provided

Table 1
Name, target sequences, 5′ position in the L gene of VHSV 07.71 (Gene Bank accession number AJ233396), number of programs which predicted the sequence and its preference order used to design small RNAs with potential interference to VHSV replication.

Name	5′ target L polymerase sequences (sense)	5′ position in the L gene	Number of programs	Preference order
o102	AAGAAAAAAGAAAGUUGUAC-UU	443	4	9th
o103	AAUUCCAAAGAAACUACAUG-UU	824	3	23th
o104	AAUGUUCACUCAACUCUCCCC-UU	53	3	37th
o105	AACGACUUCCUUCGGAAUG-UU	38	3	19th
o106	AAGACACAGAGCAAUGAGUUA-UU	250	5	1st
o107	AAUUGCUCCCUUACUGGAACU-UU	365	5	2nd
o108	AAGCUGGGAGGAUCAAGAGAU-UU	140	4	7th
o109	AAGUACACCAGGAACGACUUU-UU	26	2	78th
o110 control	AAUAGCCUGAAUUGCGGAUG-UU	–	–	–

The siRNA design software was obtained at http://i.cs.hku.hk/~sirna/software/sirna.php. It contains 12 programs from Ambion, Jack, OptiRNAi, Emboss-2.9.0, MPI principles, GenScript, Dharmacon, Qiagen, siDirect, InVitroGen, Deqor and MPI + rational. The o110 control was designed as a non-specific sequence. No significant sequence homologies of the VHSV-specific siRNA (o102–o110) could be demonstrated to the available IHNV genome sequences (Gene Bank accession numbers X73872, L40883 and X89213 corresponding to K, WRAC and Oregon 1969 strains, respectively) by virtual hybridization at 14 °C by using the Clone Manager program.

Table 2
Complete DNA sequences, corresponding to hairpins of the NS, o104, o105 and o106 siRNAs that were cloned into the pRNA-CMV3.1/puro plasmid (GenScript).

NS, non-specific (23 bp + 23 bp)
GGATCCCGTCGCTTACCGATTCAGAATGGTTGATATCCGCCATTCTGAATCGGTAAGCGACGAAGCTT
o104 (19 bp + 21 bp)
GGATCC GGGGAGAGTTGAGTGAACA TTGATATCCG TGTTCACTCAACTCTCCCCCCG AAGCTT
o105 (17 bp + 19 bp)
GGATCC CATTCCGAAGGAAGTCG TTGATATCCG CGACTTCCTTCGGAATGCG AAGCTT
o106 (19 bp + 21 bp)
GGATCC TAACTCATTGCTCTGTGTC TTGATATCCG GACACAGAGCAATGAGTTACG AAGCTT

The synthetic DNA sequences (5′–3′) were inserted into the BamHI (GGATCC) and HindIII (AAGCTT) sites of the multiple cloning site of the pRNA-CMV3.1/puro plasmid. The non-specific (NS) sequence was as provided by the manufacturer. The numbers within parentheses indicate the size of the sense and antisense oligos in base pairs (bp). The loop sequence (underlined) between the sense and the antisense siRNA sequences and the extra nucleotides *CG* (italics) were included as recommended by the manufacturer.

with a 10× ocular eye grid. The results were expressed by the ratio of the number of ffu in siRNA transfected or cloned EPC cell monolayers/number of ffu in non-transfected EPC cells × 100.

2.5. Quantification of the VHSV N protein mRNA levels by Q-RT-PCR

To perform the quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR), the primers and probe used to detect the levels of transcription of the VHSV N gene were designed with the Primer Express program included with the PE/ABD 7700 Sequence detector for nucleic acid sequence quantification (PerkinElmer/Applied Biosystems). The protein N was chosen because it is the most abundant protein and also the first to be transcribed.

The sense and antisense primers and the probe sequence were selected from the highly conserved region between nucleotides 130 and 537 of the N gene of the VHSV07.71 (accession number AJ233396). The selected primers were: 5′-TCAAGGTGACACAGGCAGTCA (sense), 5′-CCAGTTCTCTCATGGGCATCAT (antisense) and 5′-CCACGAGCATCGAGGCGGGAAT (the probe was labelled with 6-carboxyfluorescein, FAM and 6-carboxy-tetramethylrodamine, TAMRA quencher). RNA from the siRNA transfected EPC cell monolayers was extracted by using RNeasy kits from Qiagen. The cDNA was synthesized with 2–10 μg of RNA by using 50 pg of the N primer (Primer Express program), 10 mM DTT, 100 μM of each of dNTPs (Promega), 5 units of HPRI (Boehringer-Manheim), 10 μl of reverse transcriptase buffer (BRL, Gaithersburg) and 1 μl of reverse transcriptase MMLV (BRL) in a final volume of 100 μl, during 35 min at 37 °C. After adding 50 pg each of the N primers and the labelled probe, 4 units of Ampli-Taq polymerase (PerkinElmer, Weiterstadt, Germany) and water to 100 μl, the samples were heat-denatured and then amplified by 30 cycles of 1 min at 94 °C, 1 min at 59 °C and 2 min at 72 °C followed by an extension step of 10 min at 72 °C. The products were analyzed in a 7700 Sequence Detector (PerkinElmer/Applied Biosystems). The relative abundance of the corresponding mRNA was calculated from the cycle threshold (Ct) data by the formula, Ct of the non-infected EPC cells – Ct of the siRNA transfected and VHSV infected EPC/Ct of the non-infected EPC cells – Ct of the VHSV infected EPC cells × 100.

2.6. Cloning of hairpin DNA sequences in the pRNA-CMV3.1/puro plasmid

Table 2 shows the hairpin sequences cloned into the pRNA-CMV3.1/puro (GenScript, Piscataway, NJ) plasmid. Each of the three selected siRNAs (o104, o105 and o106) were converted to DNA oligos as sense and antisense sequences separated by a loop and BamHI/HindIII flanking sites added, according to Manufacturer's instructions. The oligos were then synthesized, cut with BamHI and HindIII and ligated to the corresponding plasmids. The constructed sequences were then verified by sequencing in both directions by using the recommended primers by the GenScript Manufacturer.

2.7. Transformation of EPC cells with pRNA-CMV3.1/puro-hairpin plasmids

First, EPC cells were transformed with individual pRNA-CMV3.1/puro-hairpin plasmids coding for each of the o104, o105 or o106 hairpin DNAs (Table 2). Then EPC cells were transformed with an equimolecular mixture of pRNA-CMV3.1/puro-o104 + pRNA-CMV3.1/puro-o105 + pRNA-CMV3.1/puro-o106 and clones G1B1, E2B2, G2B4 and F6B6 selected for further studies. EPC clones were also obtained by using the pRNA-CMV3.1/puro control plasmid provided by the Manufacturer (Table 2). To transfect the EPC cells, 200 ng of each of the pRNA-CMV3.1/puro-sh plasmids or 125 ng each of their mixture per well of a 96-well plate were mixed with 1 μl of fugene previously diluted in 25 μl of RPMI in 100 μl of cell culture medium containing a suspension of 500.000 EPC cells per ml. The EPC cells were then incubated at 28 °C gassed with 5% CO_2 and next day the medium was changed to fresh medium containing 2 μg of puromycin per ml. Three days later, the surviving cells were detached from the surface of the wells by using 0.05% trypsin and 2-fold serially diluted 8 times and grown in new plates. Cell colonies were detected during the next weeks and cells in those wells containing individual clones were grown and multiplied in 25 cm^2 flasks.

2.8. Assay of VHSV infected EPC clones transformed with pRNA-CMV3.1/puro-hairpin plasmids by flow cytometry

EPC cell monolayers in 24-well plates (∼500.000 cells) were infected with ∼50.000 focus forming units (ffu) of VHSV in RPMI medium containing 2% of fetal calf serum and incubated during 2 days at 14 °C. The cells were harvested with FACS buffer (100 mM Na_2HPO_4, 27 mM KCl, 17 mM KH_2PO_4, 1.3 M NaCl, pH 7.4, 0.1% bovine serum albumin, 0.01% Na_3N, 50 mM EDTA, 0.2% rabbit serum, 0.2% goat serum and 0.2% *E. coli* extract). The cell suspensions were pelleted by centrifugation, resuspended in 500 μl of FACS buffer and 250 μl of Citofix (Becton Dickinson) and permeabilized during 10 min by adding 750 μl of cold methanol. After pelleting, the cells were resuspended in FACS buffer containing 400-fold diluted anti-VHSV N MAb 2C9 (Sanz and Coll, 1992a,b) or anti-IHNV N MAb 136-3 and incubated during 1 h. Then, the cells were centrifuged again, resuspended in 100-fold diluted rabbit anti-mouse Fab′2 IgG-FITC conjugate (Nordic, Tilburg, The Netherlands) and incubated during 30 min. The cell suspensions were again centrifuged and resuspended in PBS containing 1% formaldehyde. Next day, 5000 cells were analyzed in a Beckton-Dickinson (San José, CA) FACScan apparatus.

2.9. Presence of small hairpin RNA(shRNA)-expressing DNA in G1B1, E2B2, G2B4 and F6B6 clones

The EPC clones transformed with the equimolecular mixture of plasmids described above were grown in cell culture medium containing 2 μg/ml of puromycin in 24-well plates. The corresponding cell monolayers were lysed with 120 μl/well of 50 mM KCl, 2.5 mM Mg_2Cl, 10 mM Tris pH 8.3, 150 mM EDTA, 0.45% NP40, 0.45% Tween 20 and 100 μg/ml of proteinase K during 15 min with agitation. The lysates were then transferred to a 2-ml eppendorf tubes and incubated at 55 °C during 2 h, followed by inactivation

at 95 °C during 20 min. The DNA was precipitated with ethanol and 238 mM Na acetate pH 5.2 and then washed with 70% ethanol and resuspended in 60 μl of water. The forward primer was designed inside the CMV promoter (5′TAGAAGGCACAGTCGAGG) while the reverse primer was designed inside the BGH terminator (5′CGGTAGGCGTGTACGGTG) of the vector carrying the DNA corresponding to the shRNA sequences to theoretically amplify a 192 bp fragment according to the results mimicked by using the Clone Manager program vs. 9 (Sci-Ed software, Cary, NC). PCR amplification was then performed by using 10 μM of each of the two primers, 5 U of PsP polymerase (BioRon), 200 μM dNTPs (Promega) and 5 μl of 10× buffer containing 1.5 mM of Mg_2SO_4 and 10 μl of template in a total volume of 50 μl. The mixtures were amplified by 94 °C 2 min, 30 cycles of 94 °C 1 min, 50 °C 1 min and 74 °C 1 min and a final extension step at 74 °C 10 min. Products were separated in a 1% agarose gel.

2.10. Expression of specific shRNA in G1B1, E2B2, G2B4 and F6B6 clones

Total RNA was extracted from each cell clone by the use of the miRNeasy mini-kit (Qiagen, Germany, catalog no. 217004). The QuantiMir kit (System Biosciences, Mountain View, CA) was then used to check cell clones for transcription of siRNAs. Briefly, poly-A tails were added to the RNAs by a 30 min 37 °C incubation step with poly A polymerase and annealed at 60 °C during 5 min with an oligo-dT-adaptor sequence. Products were then converted to cDNA by reverse transcription at 42 °C during 60 min. The cDNA pools of oligo-dT-anchor-tailed RNA were used as templates for quantitative SYBR green real time PCR using the premixed Brilliant SYBR Green QPCR master mix from Stratagene (La Jolla, CA), a Mx3000p Real Time PCR machine from Stratagene and prevalidated temperature cycle conditions (denaturing for 10 min at 95 °C, 40 cycles of 15 s at 74 °C, denaturing at 95 °C followed by primer annealing at 60 °C for 1 min). The forward primer used was siRNA sequence-specific and equal to the sense strand of o104, o105 or o106, respectively (Table 2). The reverse primer or adaptor specific primer at the 5′ end was supplied by the kit. Fluorescence versus number of cycles were recorded and Ct determined. Specificity of the amplified products was estimated by their temperature-dependent dissociation curve as well as by gel electrophoresis in a 2% agarose gel prestained with ethidium bromide. Data for dissociation curves were collected by measuring fluorescence while shifting the temperature of the tubes from 52 to 80 °C and holding it for 30 s at each end. Fluorescence-R′(T), the first derivative of the fluorescence of the melting curves of the products were obtained and plot. For most of the siRNAs bimodal (high and low melting temperature products) dissociation curves were obtained, reflecting specific and non-specific products which could be detected by gel electrophoresis. The high melting temperature products obtained only with the G1B1, E2B2, G2B4 and F6B6 clones peaked around 72 °C (from 66 to 76 °C), while the low melting temperature products obtained in all the samples peaked around 57 °C (from 52 to 62 °C). The non-specific products could be due to primer-dimer formation (Ma et al., 2006). Because the non-specific products were only seen when the specific template was low and because the assay has been optimized for detection of small RNAs, further optimization was not attempted. Neither sequencing nor Northern blots to further characterize the amplified products were attempted because of the small RNA amounts.

2.11. PCR amplification of Mx1

The QuantiMir kit cDNA generated from total RNA from the EPC cell clones or EPC transfected with siRNA against the protein G gene of VHSV (positive control) (Schyth et al., 2006) was used in RT-PCR reactions to estimate the level of the interferon-induced carp Mx1 gene. The forward primer 5′-ACAGAAGGAACTGGAGGCGTA and the universal reverse primer from the kit were used to amplify a fragment in the carp Mx1 (accession number AY303813). The real-time PCR reactions were run by using the premixed Brilliant SYBR Green Q-PCR master mix from Stratagene (La Jolla, CA) with 5 μl of cDNA in a final volume of 30 μl. Parallel PCR with primers for carp β-actin (accession number M24113; forward primer 5′-TCTGGCATCACACCTTCTAC) were performed with all samples as an internal control to standardize the RT-PCR results. The amplification conditions and generation of dissociation curves were performed as described above for the siRNA detection. The mRNA transcript level of Mx1 was normalized to the β-actin gene level and values expressed relative to the value of the non-infected EPC cells by using the delta delta Ct method. This meant first normalizing Mx1 Ct values in the samples to the housekeeping gene by subtracting Ct of the β-actin measurements from all Mx1 Cts. To express these values relative to the non-infected EPC cells, percentages were calculated by the formula, Ct of the non-infected EPC cells – Ct of the siRNA transfected EPC and infected with VHSV/Ct of the non-infected EPC cells – Ct of the EPC cells infected with VHSV × 100.

3. Results

3.1. Selection of possible siRNA targets in the L gene of VHSV07-71

Table 1 shows the selected siRNA sequences, their name, their position on the L gene, the number of programs which selected each siRNA sequence and their suggested order of preference. To maximize their possible interference, preferences were given to those sequences nearer the initial ATG. To minimize possible induction of cellular immune defenses (i.e.: interferon, Mx), the smaller sizes of 20–25 mer were chosen for the siRNAs. The o104 and o105 were overlapping in 6 nucleotides while the o105 and o109 had 11 overlapping nucleotides. Non-specific siRNA sequences were either designed (o110) or commercially obtained (fluorescent o121 and o122) (see Section 2). The selected sequences were then synthesized as double stranded RNA.

3.2. VHSV infection of siRNA transfected EPC cell monolayers

To estimate the possible RNA interference with VHSV infection of the selected siRNAs, EPC cell monolayers were transfected with each of the different synthetic siRNAs at concentrations lower than 2.5 μg/ml to minimize their toxicity, then infected with VHSV and the extent of infection estimated by immunodetection of the N protein of VHSV. siRNA transfection efficiencies of EPC cells were between 10 and 20% ($n = 6$) as estimated with fluorescently labelled siRNAs.

For each of the siRNA sequences, the number of N positive foci (DAB-stained foci) in the EPC cell monolayers was negatively correlated with the concentration of siRNAs with which these same cell monolayers had been transfected (Fig. 1A and B). Although control siRNA sequences (o110, o121, o122; Fig. 1A) also reduced the N positive foci 20–45% compared to the non-transfected cells, some larger but variable reductions were seen for some of the VHSV specific siRNAs at concentrations between 3 and 50 ng siRNA/well (∼70% reduction for o104 and ∼38% for o106 at 50 ng/well). In cells transfected with 100 ng of siRNA/well, o104, o105 and o106 reduced the N positive foci ∼65%, ∼45% and ∼62%, respectively, as compared to ∼45% reduction in cells transfected with control siRNAs (Fig. 1B). However, since most of those differences were not statistically significant and concentrations of 50 ng/well (500 ng/ml) are 10–50-fold more than what is normally necessary to induce gene knockdown, the observed reductions might be due to other effects. Therefore, it is not surprising that the non-specific siRNAs

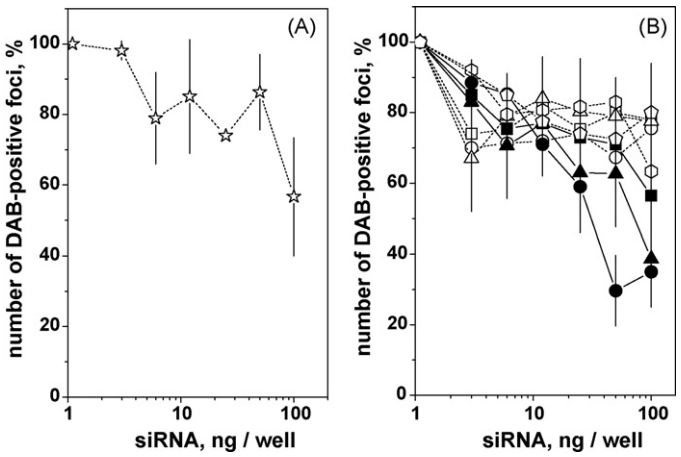

Fig. 1. Number of N-positive (stained with anti-N and DAB) foci in EPC cell monolayers transfected with different siRNA and infected with VHSV. EPC cell monolayers in 96-well plates were transfected with each of the different siRNAs (Table 1) and next day infected with 2×10^2 ffu of VSHV 07.71. After 24 h of infection, the monolayers were fixed with methanol and stained with MAb anti-N, anti-mouse IgG labelled with peroxidase and DAB. DAB-positive foci of 10–20 cells per foci were then counted and their percentage calculated by the formula, number of DAB-positive foci in siRNA transfected EPC cell monolayers/number of DAB positive foci in EPC cell monolayers × 100. The mean and the standard error of three different experiments are represented. The siRNA used were: (A) (☆) mean from non-specific siRNAs assayed independently and then averaged: o110 (sequence in Table 1), o121 and o122 (sequence in Section 2). (B) VHSV specific siRNAs: (◯) o102. (□) o103. (●) o104. (■) o105. (▲) o106. (△) o107. (☆) o108. (⬡) o109 (sequences in Table 1).

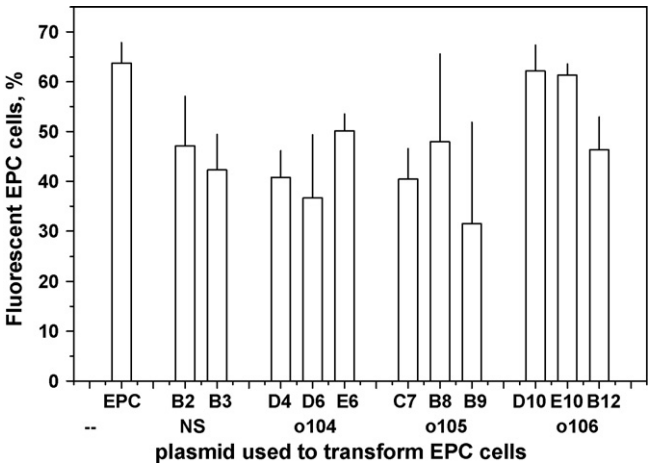

Fig. 2. Fluorescent EPC cells after VHSV infection of EPC clones transformed with each of pRNA-CMV3.1/puro-non-specific (NS), -o104, -o105 or -o106 hairpins. EPC cells were separately transfected with each the corresponding plasmids and then selected with 2 μg/ml of puromycin. After cloning by limiting dilution and grown during about 3 months, representative clones were grown in monolayers in 24-well plates and infected with VHSV. The infected clones were incubated during 2 days at 14 °C, the cells were detached, stained with Mab 2C9 specific for the N protein of VHSV and 5000 cells from each culture analyzed by flow cytometry. The mean and standard deviation of duplicates from 2 different experiments are represented. EPC, non-transfected EPC cells. B2 and B3, clones of EPC cells transformed with pRNA-CMV3.1/puro-non-specific (NS) siRNA (Table 2). D4, D6 and E6, clones of EPC cells transformed with pRNA-CMV3.1/puro-o104. C7, B8 and B9, clones of EPC cells transformed with pRNA-CMV3.1/puro-o105. D10, E10 and B12, clones of EPC cells transformed with pRNA-CMV3.1/puro-o106.

induced some degree of inhibition at high concentrations of siRNA transfection.

Similar siRNA transfection/VHSV infection experiments were then performed with o104, o105 and o106 and the mRNA of the protein N of VHSV estimated by Q-RT-PCR. By using this method, VHSV infection of EPC cells transfected with o104, o105 or o106 were reduced to 11–22% of the non-transfected infected EPC cell monolayers, while the EPC cells transfected with o102 or o110 were reduced only to 62–74% (Table 3). The other siRNA behaved similarly to the o102 or o110 (not shown).

Because that scarce evidence of significant RNA interference with VHSV infection obtained could be due to the low siRNA efficiency of transfection (siRNA transfection efficiencies of EPC cells varied between 10 and 20% ($n = 6$) as estimated with fluorescently labelled siRNAs), EPC cell lines and clones stably expressing RNAs as small hairpin siRNA (shRNA) precursors were attempted as a means to increase both the percentage of EPC cells containing siRNA and the amount of siRNA in individual cells.

Table 3
Q-RT-PCR of the mRNA of protein N of VHSV in EPC cells transfected with siRNA and infected with VHSV.

Oligos	mRNA of N, %	*n*
o102	74.7 ± 21.0	4
o104	22.5 ± 10.5	2
o105	11.7 ± 9.7	8
o106	15.6 ± 2.9	2
o110 ctrl	62.0 ± 12.3	7

The EPC cell monolayers in 96 well plates were transfected with each of the siRNA at 50 ng/well and next day infected with 2×10^2 ffu of VSHV 07.71 per well. One day later, the RNA was extracted and the mRNA of the protein N estimated by Q-RT-PCR. The mRNA synthesized was calculated from the Ct data by the formula, Ct of the non-infected EPC cells – Ct of the siRNA transfected EPC and infected with VHSV/Ct of the non-infected EPC cells – Ct of the EPC cells infected with VHSV × 100 (n = number of experiments). The other siRNAs (Table 1) were not significatively different from o102 or o110.

3.3. EPC clones transformed with individual pRNA-CMV3.1/puro plasmids coding for o104, o105 or o106 hairpins

DNA hairpins corresponding to the o104, o105 or o106 and a NS siRNAs (Table 2) were synthesized and cloned into the pRNA-CMV3.1/puro vector (GenScript), a commercial plasmid allowing for transcription of shRNAs under the control of the human cytomegalovirus promoter (CMV) and coding for the puromycin resistance gene (puro) to allow puromycin selection of transfected cells. EPC cells were transfected with each of the pRNA-CMV3.1/puro-hairpins, cell lines selected with puromycin and 2–3 clones for each siRNA obtained by limiting dilution: NS (B2 and B3 clones), o104 (D4, D6 and E6 clones), o105 (C7, B8 and B9 clones) or o106 (D10, E10 and B12 clones). The clones were maintained in the presence of puromycin and grown for several months before any other test.

Fig. 2 shows the number of VHSV-infected EPC cells (fluorescent cells) detected by flow cytometry after their staining with anti-VHSV N MAb 2C9. With an VHSV infection of 63.7% of the non-transfected EPC cells (EPC bar in Fig. 2), the number of infected cells transformed with pRNA-CMV3.1/puro-NS were 47.1 and 42.3% in NS clones B2 and B3, respectively, while o104 clones D4, D6 and E6 showed 40.8, 36,6 and 50.1% of infected cells, respectively, o105 clones C7, B8 and B9 showed 40.4, 47.9 and 31.5% of infected cells, respectively, and o106 clones D10, E10 and B12 showed 62.1, 61.3 and 46.3% of infected cells, respectively. Accordingly no specific reduction of VHSV infection was obtained when any of these constructs were used to transform EPC cells individually.

3.4. EPC clones co-transformed with a mixture of pRNA-CMV3.1/puro plasmids coding for o104, o105 or o106 hairpins

Because preliminary results showed that EPC cell lines co-transformed with an equimolecular mixture of pRNA-CMV3.1/

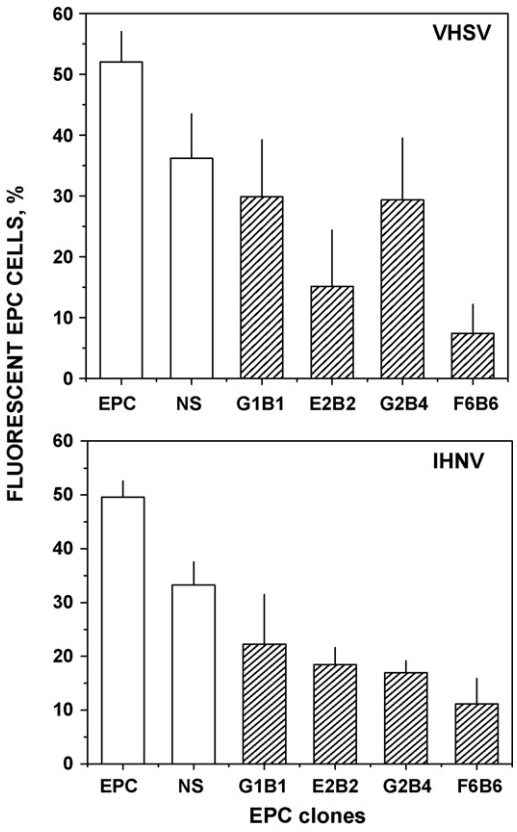

Fig. 3. Infected (fluorescent) EPC cells after VHSV or IHNV infection of EPC clones G2B4, F6B6, G1B1 and E2B2. EPC cells were transfected with an equimolecular mixture of pRNA-CMV3.1/puro-o104, pRNA-CMV3.1/puro-o105 and pRNA-CMV3.1/puro-o106 plasmids and then selected with 2 μg/ml of puromycin. After cloning by limiting dilution and continuous growth during 3–6 months, 4 representative clones were subcultured in monolayers in 24 well plates (~500.000 cells per well) and infected with VHSV or IHNV. The infected cell cultures were incubated during 2 days at 14 °C. Cells were then detached, stained with Mab 2C9 specific for the N protein of VHSV or Mab 136-3 specific for the N protein of IHNV and 5000 cells per point analyzed by flow cytometry. The mean and standard deviation of duplicates from three (VHSV) and two (IHNV) different experiments are represented. EPC, non-transfected EPC cells. NS, EPC cells transformed with pRNA-CMV3.1/puro-non-specific siRNA (Table 2). G1B1, E2B2, G2B4 and F6B6, selected clones transformed with pRNA-CMV3.1/puro-o104 + pRNA-CMV3.1/puro-o105 + pRNA-CMV3.1/puro-o106.

puro-o104, pRNA-CMV3.1/puro-o105 and pRNA-CMV3.1/puro-o106 had a higher reduction of VHSV infectivity than the clones obtained with the individual plasmids (data not shown), clones were derived from the EPC cell lines obtained after co-transformation with such mixtures. In order to do that, EPC cell lines co-transformed with the equimolecular mixture of the above mentioned plasmids were cloned by limiting dilution in the presence of puromycin and clones G1B1, E2B2, G2B4 and F6B6 were selected for further studies.

Fig. 3 VHSV shows the number of VHSV infected EPC cells by flow cytometry after anti-VHSV N Mab 2C9 staining of the VHSV infected EPC clones mentioned above. With a 52.0% of VHSV infection of the non-transformed EPC cells, the number of infected cells were of 36.2, 29.8, 15.1, 29.3 and 4.7% in the NS, G1B1, E2B2, G2B4 and F6B6 clones, respectively.

Fig. 3 IHNV shows the number of IHNV infected EPC cells by flow cytometry after anti-IHNV N Mab 136-3 staining of the IHNV infected EPC clones. With a 49.6% of IHNV infection of the non-transformed EPC cells, the number of infected cells were of 33.2, 22.2, 18.4, 16.9 and 11.1% in the NS, G1B1, E2B2, G2B4 and F6B6 clones, respectively.

EPC cell clones expressing VHSV specific siRNAs, were also able to inhibit replication of the heterologous IHNV to similar extents (Fig. 3). While some sequence-specific inhibition of VHSV was expected because of the complementarity of the siRNA sequences to their L gene, no significant sequence homology of the o104, o105 or o106 could be demonstrated to the IHNV genome sequences (Gene Bank accession numbers X73872, L40883 and X89213) by virtual hybridization at 14 °C by using the Clone Manager program (data not shown). However, inhibition could be explained by the siRNAs targeting the IHNV if the shRNAs could be working as microRNAs (miRNAs) where only the 5′ end 2–8 first nucleotides have to be complementary to the L gene target in order to interfere. To examine this possibility, the program Miranda (http://www.rnaiweb.com/RNAi/MicroRNA_Tools_software/MicroRNA) was used to search for microRNA target sites of the o104, o105 and o106 sequences in the 3 available genomic IHNV sequences by using a low energy threshold (−14 kcal/mol) in order to find as many potential targets as possible. However, no potential sites were found for o104 or o106 except for one very low energy of o106 in IHNV X73872 (which was not found in IHNV L40883 and X89213). However, o105 was predicted by Miranda to bind the same potential target site in the three IHNV (−18 to −20 kcal/mol energy for binding), data which could explain, at least, some of the heterologous interference observed.

The flow cytometry results showed that expression of NS siRNAs in the NS clones in itself slightly reduced VHSV and IHNV replication (30.4–33.1% inhibition) (Fig. 3), which was also experienced in the siRNA transfection experiments with VHSV as estimated by using the immunostaining assay in cell monolayers (20–45% inhibition depending on siRNA concentration) (Fig. 1). Because the lack of specificity could be due to residual anti-VHSV activity of some puromycin traces remaining in any of the transformed EPC clones including the NS (despite those being infected in the absence of puromycin), the clones were grown in the absence of puromycin during 3 passages and then assayed for VHSV infection by using the immunostaining assay in cell monolayers. The results showed that the NS clones whether they were grown in the absence or in the presence of puromycin showed again a slightly non-specific siRNA sequence inhibition of VHSV infection with respect to the non-transformed EPC cells (19.7–21.7% inhibition) (Table 4). These results also showed that the EPC clones were stable in the absence of puromycin. Similar results were obtained whether the VHSV infectivity in the clones was assayed by flow cytometry (Fig. 3) or by immunostaining the cell monolayers (Table 4), and confirmed that the F6B6 clone was strongly resistant to VHSV infection (Fig. 3 and Table 4).

Table 4
DAB-positive foci (ffu) of EPC cells after VHSV infection of EPC clone cell monolayers of NS, G2B4, F6B6, G1B1 and E2B2 grown after 3 passages in the presence and in the absence of puromycin.

EPC clones	ffu, % of control	
	+Puromycin	−Puromycin
NS	78.3 ± 4.8	80.3 ± 4.7
G1B1	74.0 ± 16.8	55.6 ± 14.8
E2B2	26.1 ± 15.5	23.5 ± 12.9
G2B4	45.9 ± 1.0	26.5 ± 1.5
F6B6	0.3 ± 0.1	0.4 ± 0.1

EPC clone cells were passaged 3 times during 2 months in the presence or in the absence of 2 μg/ml of puromycin. The resulting EPC clone cell monolayers in 96-well plates were infected with 1.5×10^3 ffu of VSHV 07.71 per well in the absence of puromycin. After 24 h of infection, the monolayers were fixed with methanol and stained with MAb anti-N, anti-mouse IgG labelled with peroxidase and DAB. DAB-positive foci of 10–20 cells per foci (ffu) were then counted and their percentage calculated by the formula, number of ffu in EPC clone cell monolayers/number of ffu in EPC cell monolayers (control) × 100. The mean and the standard deviations of 2–3 different experiments each in triplicate were calculated.

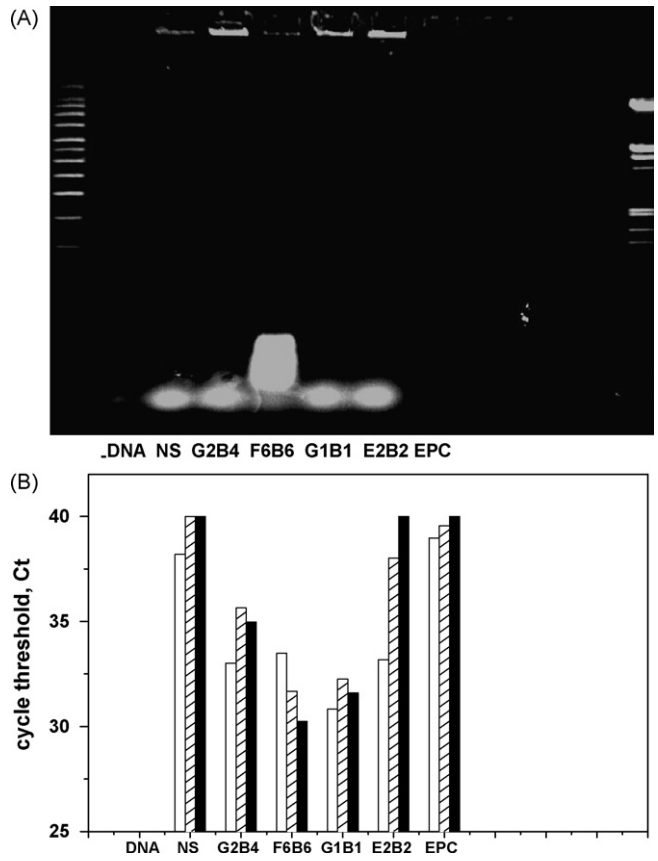

Fig. 4. Presence of small hairpin (sh) sequences in the DNA by PCR (A) and detection of o104, o105 and o106 siRNAs in the RNA by RT-PCR (B) in the EPC clones. (A) DNA extracted from EPC clones grown in cell culture medium containing 2 μg of puromycin during several months, was amplified by PCR to a ~200 bp fragment containing any sh DNAs and flanked amplified sequences. Supercoiled DNA 2–16 kbp markers and single stranded DNA 0.5–21 kbp markers are shown to the left and to the right of the gel, respectively. (B) RNA extracted from EPC clones grown in cell culture medium containing 2 μg of puromycin during several months was poly-A tailed, oligo-dT-adaptor hybridized, cDNA converted and o104, o105 or o106 specific real time PCR-amplified. The o104, o105 and o106 siRNA expression levels were estimated by their comparative cycle thresholds (Ct). Empty bars, o104. Hatched bars, o105. Black bars, o106.

3.5. Presence of specific o104, o105 and o106 DNA and RNA sequences in the G1B1, E2B2, G2B4 and F6B6 EPC clones

The presence of specific DNA sequences corresponding in size (~200 bp) to the cloned sh DNAs plus the flanked amplified sequences was shown by PCR amplification of the DNA extracted from each of the EPC clones. Thus, Fig. 4A shows the PCR amplified bands corresponding to the expected sizes in NS, G1B1, E2B2, G2B4 and F6B6 clones, while no bands could be obtained by amplifying DNA from EPC cells. Smearing corresponding to several bands of 2–3-fold higher molecular weight appeared in F6B6, most probably due to the presence of head-to-head or head-to-tail polymers (concatemers) of the DNAs corresponding to the shRNA sequences.

The expression level (Ct) (Fig. 4B) and the presence of RNA specific products (high melting temperature dissociation curves) (Fig. 5) of the siRNA sequences in the total RNA from each of the EPC clones were then estimated by poly-A tailing of total RNA, oligo-dT-adaptor hybridization, cDNA conversion and o104, o105 or o106 specific real time PCR. Most of the Cts were around 32–33, with expression of o104, o105 and o106 in G1B1 and F6B6 being 2–3 Cts lower than in the rest (Fig. 4B). High melting 70–72 °C amplified products were expressed by G1B1 with o104, o105 and o106, while F6B6 only expressed o105 and o106 (Fig. 5). Non-specific products with melting temperatures around 56 °C were seen as most of the products in the EPC and NS control clones, similarly to what was found for E2B2 and G2B4 clones. Since the non-specific low melting temperature products could be a strong competitor for the specific products, the Cts estimations are difficult to interpret, except for the G1B1 and F6B6 clones. Further, the expression levels were considered low, as only the strongest expressing clones F6B6 and G1B1 (Ct around 31) had low levels of most non-specific products.

The expression of o106 could be detected in G1B1 and F6B6 clones but not in the G2B4 clone by agarose gel electrophoresis (data not shown), thus verifying that both the specific and non-specific products predicted from the melting curves are real products. Further, the electrophoretic results showed that the product size of those specific products were about 100 bp, corresponding to shRNA specific PCR products (small hairpins plus adaptor lengths).

3.6. Induction of Mx in the NS, G1B1, E2B2, G2B4 and F6B6 EPC clones

None of the NS, G1B1, E2B2, G2B4 and F6B6 clones expressing different levels of endogenous siRNAs (Figs. 4B and 5) showed any significant upregulation of carp Mx1 expression compared to the non-transfected EPC cells (data not shown). In contrast, transfection of EPC cells with exogenous siRNA to the protein G gene of VHSV showed a ~20–400-fold increase in carp Mx levels, confirming earlier work (Schyth et al., 2006). Since the lack of Mx expression of the EPC clones might reflect the presence of functional interferon, the conclusion that in G1B1 and F6B6 (the clones with a higher expression of endogenous siRNAs), the siRNA did not induce the antiviral interferon system requires further investigation.

4. Discussion

Transfection of EPC cells with 8 synthetic siRNAs (o102–o109) targeted to the L polymerase gene sequence of VHSV07.71 followed by VHSV infection, failed to demonstrate a significant reduction of VHSV infection (perhaps with the exception of o104 and o106 siRNAs). Since VHSV detection by immunodetection and/or Q-RT-PCR required infection of ~100.000 EPC cells with 100–150 VHSV ffu (1–1.5 ffu per 1000 cells), the inhibition of VHSV infection could not be complete (maximally observed was 30% at 100 ng of siRNA/well). Therefore, the results obtained could be due to low and variable siRNA transfection efficiencies of EPC cells (10–20% as estimated with fluorescently labelled siRNAs). Direct siRNA microinjection in fish cells seems to be more efficient than their transfection (Gruber et al., 2005) and the production of endogenous siRNA from eukaryotic plasmids with shRNA-generating DNA sequences has been demonstrated (Xia et al., 2003). We obtained transformed EPC cell lines and clones stably producing endogenous small hairpin RNA (shRNA) to increase the percentage of EPC cells containing siRNA in the clones and the amount of siRNA in individual cells.

Because inhibition of VHSV infection was not observed by using EPC clones obtained by transfecting with individual siRNAs, four EPC clones named G1B1, E2B2, G2B4 and F6B6 were generated by co-transforming EPC cells with an equimolecular mixture of the plasmids containing the corresponding sh DNAs for the o104, o105 and o106 sequences and a NS sequence (Table 2). Evidence for the presence of the corresponding DNA sequences and their expression of, at least, some of the siRNAs per clone, could then be demonstrated (Figs. 4 and 5) indicating that this method may be useful to generate endogenous siRNA expressing fish cells where transfection of exogenous siRNAs is not required to perform experiments.

Inhibition of VHSV or IHNV infection with respect to non-transformed EPC cells was observed in the NS clone (non-specific sequence inhibition) as well as in the clones expressing the specific

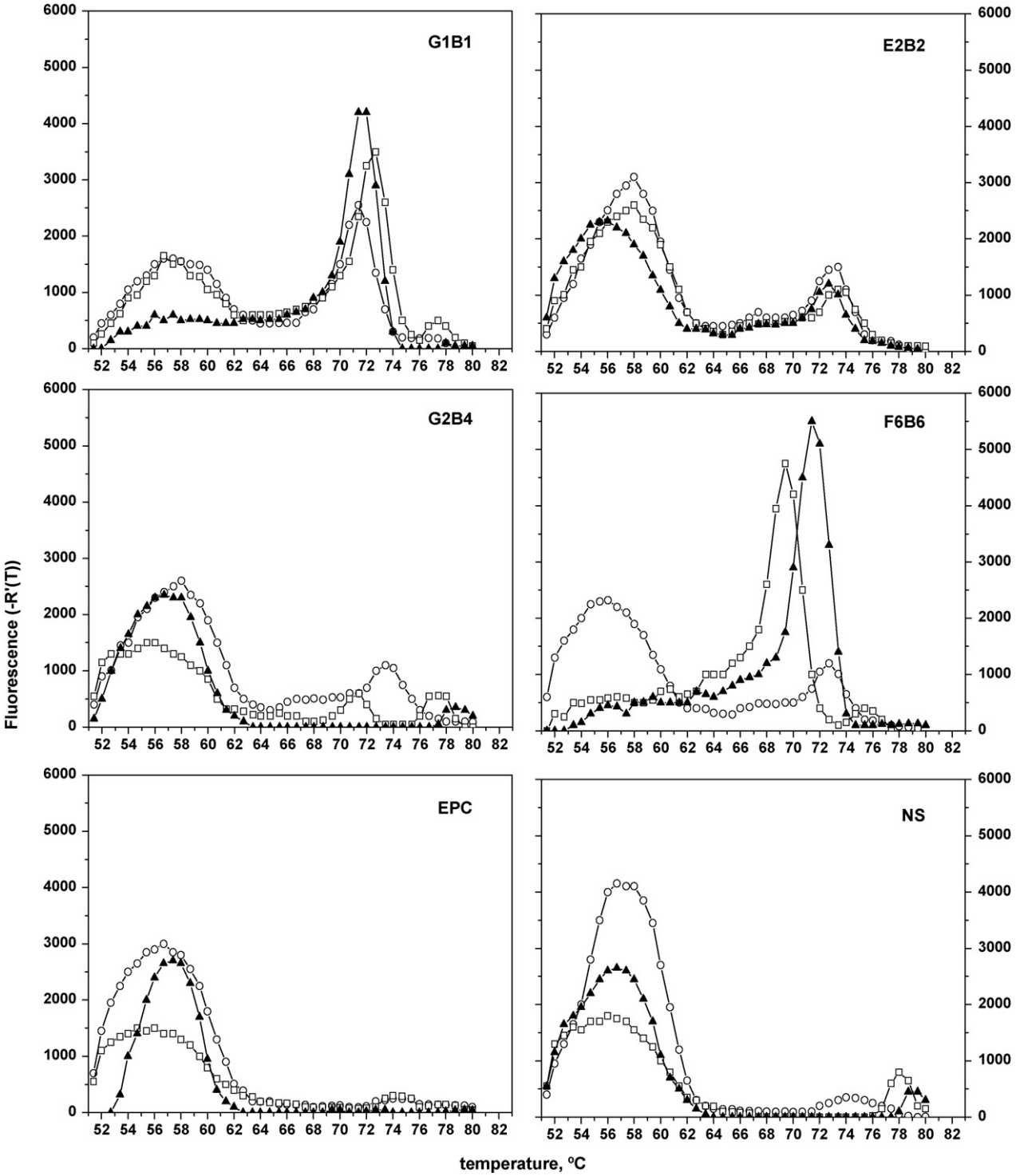

Fig. 5. Dissociation curves of the RT-PCR amplified o104, o105 and o106 siRNAs from the EPC clones. The specific transcriptional products with melting temperatures around 72 °C and non-specific products with melting temperatures around 56 °C (melting dissociation curves) were estimated in the RT-PCR amplified products shown in Fig. 4B. Fluorescence-R′(T), the first derivative of the fluorescence of the melting curves of the RT-PCR products were plot from 52 to 80 °C. The corresponding melting dissociation curve symbols are: (◯) o104. (□) o105. (▲) o106.

siRNAs (virus-unspecific inhibition), thus implying that the endogenous siRNAs sequences induced a general antiviral mechanism. Such had previously been shown to be due to Mx/interferon induction in fish cells and mammalian cells transfected with exogenous siRNAs (Schyth et al., 2006, 2007), but in this case an increased level of the interferon-induced Mx1 gene could not be demonstrated, perhaps due to the endogenous source of siRNAs. Alternatively, the unspecificity seen, might be due to some interference of the double stranded shRNAs with the VHSV/IHNV double stranded intermediate replication forms or to some residual anti-VHSV activity of puromycin remaining in any of the transformed clones but no evidence could be obtained for the latter (Table 4).

When the EPC cell clones G1B1, E2B2, G2B4 and F6B6 were infected with VHSV or IHNV, a further decrease in VHSV but also of IHNV infection could be detected, specially in clones E2B2 and F6B6 (Fig. 3). However these results were difficult to correlate with

their siRNA expression patterns (Figs. 4B and 5), thus showing again that the observed inhibition might not be sequence-specific. However, some sequence-specific inhibition might still remain since it was predicted by the Miranda program that o105 might work as a potential miRNA targeting IHNV as well as VHSV, thereby providing a possible explanation of why IHNV infection could also be reduced, at least in F6B6. The reason why G1B1 was not inhibiting either VHSV or IHNV significantly compared to the NS control clone (Fig. 3) could be due to the slightly lower expression of o105 and o106 in this clone compared to F6B6 (Fig. 5). However, we could not explain why E2B2 and G2B4 clones showed slightly higher antiviral effects against VHSV and VHSV/IHNV, respectively, compared to the NS control clone.

The F6B6 clone, the clone that showed the highest resistance to viral infection (Fig. 3 and Table 4) was expressing more o105 and o106 of all clones (Fig. 5) and also contained higher molecular weight DNA inserted into the genome (possible concatemers?) (Fig. 4A). However, the F6B6 cell monolayers seem to be under much more stress than any of the other EPC clones since more cells were rounded up in these cultures than in any other (not shown), while cells adhering to the well surface were looking healthy. Therefore, the expression of siRNA sequences in F6B6 might be interfering with normal EPC cell metabolism causing a possible cellular toxicity that could also explain why they interfered with VHSV/IHNV replication. The reason for the possible toxicity could be that this clone had a higher expression of siRNAs, causing its miRNA machinery to be occupied by siRNAs excess and hence non-functional with respect to the cells' own household mechanisms or to the expression of larger concatemer RNAs.

5. Conclusions

Despite the search for optimal conditions to obtain viral sequence-specific siRNA interference in EPC cells, the inhibition of VHSV *in vitro* infection in EPC clones expressing detectable VHSV L protein-specific siRNAs was variable among clones, non-Mx-dependent and non-specific for the virus. The siRNA expression in some of the EPC clones shows that we have a system for generating fish cell specific siRNA, most probably matured by the fish's own RNAi apparatus including Drosha and Dicer, where we do not need to rely on using chemically synthesized exogenous siRNAs. Further, if the system can be optimized for a higher but non-toxic expression of siRNAs (detection of non-specific products indicating that expression levels were low), such as by using stronger (Zenke and Kim, 2008) or Pol III-driven shRNA promoters, the antiviral effect on the EPC cell clones could probably become more pronounced and reproducible.

Acknowledgements

Thanks are due to Ana Rocha for technical assistance on running PCRs and Lasse Bøgelund Kristensen for technical assistance on running the PCR for small RNAs. This work was supported by projects AGL2005-00339/ACU, AGL2008-03519-CO4 and CSD00C07-00002 funded by the program Consolider-Ingenio 2010, from the Spanish Ministerio de Innovación y Ciencia and from the Danish Research Council for Technology and Production Sciences grant numbers 26-03-0059 and 274-05-0585, the Lundbeck Foundation grant number 52/03 and the European Commission through contract FP6007103 IMAQUANIM.

References

Baltayiannis, G., Filis, S., Tsianos, E., Karayiannis, P., 2005. Small interfering RNA(siRNA)-mediated inhibition of hepatitis C virus replication using bicistronic vector in vivo (cell line Huh-7) and in vitro. J. Hepatol. 42, 154.

Barik, S., 2004. Control of nonsegmented negative-strand RNA virus replication by siRNA. Virus Res. 102, 27–35.

Basurco, B., Sanz, F., Marcotegui, M.A., Coll, J.M., 1991. The free nucleocapsids of the viral haemorrhagic septicaemia virus contain two antigenically related nucleoproteins. Arch. Virol. 119, 153–163.

Caplen, N.J., 2003. RNAi as a gene therapy approach. Expert Opin. Biol. Ther. 3, 575–586.

Colbère-Garapin, F., Blondel, B., Saulnier, A., Pelletier, I., Labadie, K., 2005. Silencing viruses by RNA interference. Microbes Infect. 7, 767–775.

Coll, J.M., 1995. The glycoprotein G of rhabdoviruses. Arch. Virol. 140, 827–851.

Gruber, J., Manninga, H., Tuschl, T., Osborn, M., Weber, K., 2005. Specific RNAi mediated gene knockdown in zebrafish cell lines. RNA Biol. 2, 101–105.

Heike, S., Egbert, M., Mettenleiter, T.C., 1999. Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. Virus Genes 19, 59–65.

Korf, M., Jarczak, D., Beger, C., Manns, M.P., Krüger, M., 2005. Inhibition of hepatitis C virus translation and subgenomic replication by siRNAs directed against highly conserved HCV sequence and cellular HCV cofactors. J. Hepatol. 43, 225–234.

Kurath, G., Higman, K.H., Björklund, H.V., 1995. The NV genes of fish rhabdoviruses: development of RNase protection assays for rapid assessment of genetic variation. Vet. Res. 26, 477–485.

LaPatra, S.E., Lauda, K.A., Jones, G.R., 1994. Antigenic variants of infectious hematopoietic necrosis virus and implications for vaccine development. Dis. Aquat. Organisms 20, 119–126.

LeBerre, M., De Kinkelin, P., Metzger, A., 1977. Identification sérologique des rhabdovirus des salmonidés. Bull. Office Int. Epizooties 87, 391–393.

Li, T., Zhang, Y., Fu, L., Yu, C., Li, X., Li, Y., Zhang, X., Rong, Z., Wang, Y., Ning, H., Liang, R., Chen, W., Babiuk, L.A., Chang, Z., 2005. siRNA targeting the leader sequence of SARS-CoV inhibits virus replication. Gene Ther. 12, 751–761.

Lorenzen, N., LaPatra, S.E., 2005. DNA vaccines for aquacultured fish. Rev. Sci. Technol. 24, 201–213.

Lorenzo, G., Estepa, A., Coll, J.M., 1996. Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicemia with anti-nucleoprotein monoclonal antibody. J. Virol. Methods 58, 1–6.

Ma, H., Shied, K.J., Chen, G., Qiao, X.T., Chuang, M.Y., 2006. Application of real time polymerase chain reaction (RT-PCR). J. Am. Sci. 2, 1–5.

Novina, C.D., Murray, M.F., Dykxhoorn, D.M., Beresford, P.J., Riess, J., Lee, S.K., Collman, R.G., Lieberman, J., Shankar, P., Sharp, P.A., 2002. siRNA-directed inhibition of HIV-1 infection. Nat. Med. 8, 681–686.

Purcell, M.K., Hart, S.A., Kurath, G., Winton, J.R., 2006. Strand-specific, real-time RT-PCR assays for quantification of genomic and positive-sense RNAs of the fish rhabdovirus, infectious hematopoietic necrosis virus. J. Virol. Methods 132, 18–24.

Rocha, A., Ruiz, S., Coll, J.M., 2004. Improvement of transfection efficiency of epithelioma papulosum cyprini carp cells by modification of their cell cycle and use of an optimal promoter. Mar. Biotechnol. 6, 401–410.

Sanz, F., Coll, J.M., 1992a. Detection of viral haemorrhagic septicemia virus by direct immunoperoxidase with selected anti-nucleoprotein monoclonal antibody. Bull. Eur. Assoc. Fish Pathol. 12, 116–119.

Sanz, F.A., Coll, J.M., 1992b. Detection of hemorrhagic septicemia virus of salmonid fishes by use of an enzyme-linked immunosorbent assay containing high sodium chloride concentration and two noncompetitive monoclonal antibodies against early viral nucleoproteins. Am. J. Vet. Res. 53, 897–903.

Schyth, B.D., Lorenzen, N., Pedersen, F.S., 2006. Antiviral activity of small interfering RNAs: specificity testing using heterologous virus reveals interferon-related effects overlooked by conventional mismatch controls. Virology 349, 134–141.

Schyth, B.D., Lorenzen, N., Pedersen, F.S., 2007. A high throughput in vivo model for testing delivery and antiviral effects of siRNAs in vertebrates. Mol. Ther. 15, 1366–1372.

Wagner, R.R., 1987. The Rhabdoviruses. Plenum Press, NY, 543.

Xia, X.G., Zhou, H., Ding, H., Affar, E.B., Shi, Y., Xu, Z., 2003. An enhanced U6 promoter for synthesis of short hairpin RNA. Nucleic Acids Res. 31, e100.

Zenke, K., Kim, K.H., 2008. Novel fugu U6 promoter driven shRNA expression vector for efficient vector based RNAi in fish cell lines. Biochem. Biophys. Res. Commun. 371, 480–483.